(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 October 2003 (23.10.2003)

PCT

(10) International Publication Number
WO 03/086612 A1

(51) International Patent Classification[7]: B01J 19/00, C07B 61/00

(21) International Application Number: PCT/GB03/01615

(22) International Filing Date: 10 April 2003 (10.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0208218.8 10 April 2002 (10.04.2002) GB
0217722.8 31 July 2002 (31.07.2002) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/086612 A1

(54) Title: METHOD OF PRODUCING DYNAMIC COMBINATORIAL LIBRARIES

(57) Abstract: The present invention relates to a method of producing a dynamic combinatorial library of chemical compounds. In particular, the present invention relates to a method of producing a dynamic combination of compounds containing at least one chemical entity having an arrangement of functional groups that renders it capable of binding to a molecular target, said entity being generated through the interaction of enzymes with appropriate substrates under conditions suitable for both formation and cleavage of chemical bonds. The presence of the molecular target results in an equilibrium redistribution in favour of the entity or entities interacting with said molecular target. Thus, the entity or entities interacting with the molecular target, in particular the entity or entities interacting most strongly with the target, may be identified and, if necessary, characterised.

METHOD OF PRODUCING DYNAMIC COMBINATORIAL LIBRARIES

The present invention relates to a method of producing a dynamic combinatorial library of chemical compounds. In particular, the present invention relates to a method of producing a dynamic combination of compounds containing at least one chemical entity having an arrangement of functional groups that renders it capable of binding to a molecular target, said entity being generated through the interaction of enzymes with appropriate substrates under conditions suitable for both formation and cleavage of chemical bonds.

The use of combinatorial chemistry to accelerate the identification of new chemical entities with desirable properties is well established. For example, in drug discovery, large collections of compounds (combinatorial chemical libraries) are often synthesised very quickly using techniques collectively called 'combinatorial chemistry'. These techniques, which rely upon the combination of chemical 'building blocks' such as substrates and reagents, can include the parallel synthesis of compound libraries using automated and non-automated methods, and may employ both solution phase and solid phase chemistry. Such combinatorial chemical libraries include peptide libraries (see, for example, US Patent No. 5,010,175), carbohydrate libraries (see, for example, Liang et al., (1996) Science, vol. 274, p.p.1520-1522), and small organic molecule libraries (see, for example, Baum et al, (1993), Chem and Eng News, Jan 18, p33). Equipment and devices for the preparation of combinatorial chemical libraries are commercially available (see, for example, Advanced Chem Tech, Louisville, KY, USA; Applied Biosystems, Foster City, CA, USA). Libraries so created may be collections of discrete, individual compounds or may consist of collections of mixtures of compounds. After a library has been created, the compounds or mixtures are screened for useful properties. Pharmaceutically useful properties may be identified through a basic screen developed to assay the ability of compounds to bind to a molecular target, which may be natural or synthetic, and may employ a receptor, an enzyme, a peptide, an enzyme substrate, a nucleic acid, a carbohydrate, a lipid or other molecule having an affinity for a ligand. If mixtures of compounds are screened, a deconvolution process is often necessary to identify the components of a mixture that are responsible for any observed activity in the screening process. This can prove difficult to achieve in practice.

Initial chemical approaches to the production of libraries by conventional techniques may be led by a random approach to structure design, computational chemistry and predictive modelling, analogue design based on compounds with known structure and activity, medicinal chemistry intuition, or combinations of these approaches. The libraries of compounds so produced are then screened for activity against targets of interest. These targets, as described above, can include enzymes and receptors, whole cells and organisms, and testing can be carried out in vitro or in vivo. Usually, the probability of finding active compounds with desirable properties from such large libraries is low, particularly where mixtures of compounds are screened, and the suitability of such compounds as drug development candidates is not good. It is often then necessary to develop new follow-up libraries of compounds with structures related to the initial active 'hit' compounds and that are predicted to have more desirable properties, and then to test these for activity. This process is often termed 'lead optimisation'. In this manner, drug discovery using combinatorial chemistry requires an iterative or evolutionary approach. Similar discovery models based on the application of traditional combinatorial chemistry are used in other chemical and biological fields of relevance to industry and commerce, for example, in the discovery of new compounds for use in agriculture as herbicides, pesticides and insecticides, and in industrial catalysis.

One drawback of existing methods of combinatorial library synthesis pertains to the step of the creation of compound variants. Traditionally, compound variants are generated by the use of conventional chemical synthesis procedures. This involves a number of steps wherein the basic chemical approach to the compound types of interest is first worked out utilising appropriate substrates and reagents. At this stage, reaction conditions are developed to enable the synthesis to be carried out by solution or solid phase chemistry, or a combination of both where some individual steps may be carried out on a solid support and others in solution. The conditions of synthesis developed are usually such as to produce high yields by driving reactions to completion under essentially irreversible conditions. This step is then usually followed by an investigation of the scope for the reaction so designed in terms of the variety of substrates and reagents that can be used to generate the required numbers and types of compounds. This step is then followed by combinatorial chemical library synthesis using an appropriate range of reagents and substrates and a suitable type of equipment.

Thus, the generation of compound variants requires large numbers of substrates and reagents, is time-consuming and requires huge amounts of work. Others such as Lehn (European Patent Application No. 1 130 009 A1) have sought to avoid this particular limitation to an extent through the process of preparing 'dynamic' combinatorial libraries. A dynamic combinatorial library can be visualised as a collection of compounds that can interconvert by a series of equilibrium processes to produce a mixture of compounds where all combinations possible through such an equilibration process are present. However, the components of such dynamic libraries are made from building blocks that are themselves complex to construct, and a further limitation is that the building blocks used must be connected by linkers that are amenable to dynamic exchange and the chemical scope of such linkers is limited.

A further drawback of these known methods is that the chemical synthesis methods themselves that are used for the production of large compound collections are unable to favour or to distinguish active compounds from inactive compounds and often produce only diminishingly small quantities of active compounds. This can lead to difficulties in finding active compounds, in compound isolation, purification, characterisation and screening against the intended target.

The usefulness of the compound libraries is largely determined by the quality and relevance of information used to direct compound and library design and by the quantities of individual compounds available for screening. For this reason, the 'hit' rate from such libraries is usually low. Others such as Lehn have sought to avoid these particular limitations to an extent through the process of preparing 'dynamic' combinatorial libraries in the presence of a molecular target (EP 1 130 009 A1). The presence of the molecular target is said to cause the composition of such a dynamic equilibrium library to be driven towards that favouring compounds that bind to the target, if such compounds are present in the dynamic equilibrium. Since a dynamic combinatorial library can be visualised as a collection of compounds that can interconvert by a collection of equilibrium processes, any library member that can be stabilised or removed from the equilibrium composition, for example as a result of binding to a molecular target added to the equilibrium mixture, will have its concentration amplified as a result of the thermodynamic redistribution of the equilibrium in favour of that compound. Although this approach has demonstrated certain advantages over traditional methods, it is limited in scope by the narrow range

of reaction conditions and suitable chemical functionalities that can allow the reversible bond formation necessary to establish a truly dynamic combinatorial library of interconverting compounds.

A further drawback of existing methods for compound library production, especially when the compounds so produced are intended to bind with complex biological targets such as proteins and nucleic acids, is the difficulty of producing libraries of compounds having defined stereochemistry and high stereochemical purity. It will be understood that the chiral nature of most biological targets is of considerable consequence and that optical isomers can exhibit very different binding of the target and therefore different biological activity because of this. Therefore, a method of producing combinatorial chemical libraries having single enantiomers and diastereoisomers or enrichment in such compounds is desirable.

The present invention has been made from a consideration of the above referenced problems with prior art methods. The novel approach of the present invention relies on the establishment of a dynamic combinatorial chemical library consisting of a collection of compounds that can interconvert by the establishment of a series of equilibrium processes brought about through the mediation of enzymes. Further, through establishing this dynamic equilibrium in the presence of a suitable molecular target, the concentration of those compounds having a higher degree of interaction with the molecular target will be amplified, thus driving the composition of the collection of compounds towards those having useful properties. This process relies on the reversible interconversion of members of the compound collection thus enabling the continuous generation of all possible combinations of components and thereby making available all structural and interactional features that these combinations may need in order to interact with the molecular target. This is followed by selection of the component among all those possible that possesses the features most suitable for formation of the optimal interaction with the molecular target, by recruiting the correct partners from the set of those available. The degree of completeness of the set of compounds in such a multi-component system is a combinatorial process with a synthesis and selection procedure directed by the kinetic and thermodynamic parameters imposed by the nature of the components and their interactions.

Thus, and in accordance with a first aspect of the present invention there is provided a method of producing a dynamic combinatorial library of compounds in the

presence of a molecular target capable of interaction with at least one functionality, said method comprising the following steps:

(i) providing a compound or a plurality of compounds each having at least one functionality, wherein said compound or plurality of compounds is susceptible to modification via at least one enzyme-catalysed reaction whereby such modification results in the formation of at least one entity having an arrangement of functionalities that allows it to interact with said molecular target;

(ii) mixing said compound or plurality of compounds with at least one enzyme and maintaining the resulting mixture under conditions that allow reversible bond formation and cleavage;

(iii) introducing said molecular target to said mixture and further maintaining said mixture under conditions that allow an equilibrium redistribution in favour of the entity or entities interacting with said molecular target; and

(iv) identifying which entity or entities interact with said molecular target.

It will be appreciated that an advantage of this aspect of the present invention arises from establishment of the dynamic combinatorial library in the presence of the molecular target, which leads to a redistribution of the thermodynamic equilibrium in favour of those entities produced that bind most strongly to the target, concurrently integrating synthesis and screening and thereby reducing the time for the discovery of compounds having useful properties. Redistribution of the thermodynamic equilibrium results in a greater degree of amplification of the entity or entities interacting most strongly with the molecular target, thus allowing said entity or entities to be identified. Clearly, the entity or entities interacting most strongly with the target of interest will typically be the most useful of the entities formed, and it is an advantage of the method of the present invention that the entity or entities interacting most strongly with the molecular target may be readily identified.

The advantages of using enzymes, either natural or synthetic, in order to synthesise compounds and to establish a dynamic combinatorial library of compounds are numerous. For example, enzymes are known to accelerate the rate of reactions and to be able to operate reversibly under mild conditions. Additionally, enzymes can be highly stereoselective for substrates in reactions that they catalyse. Importantly, the scope of bond formation and cleavage reactions that enzymes can catalyse and that are useful in compound library synthesis are both diverse and numerous and include:

formation and cleavage or hydrolysis of ester linkages, amides, phosphates, sulphates, lactones, nitriles, epoxide, anhydride, glycosidic and C-C bonds; the transfer of groups such as aldehydic, ketonic, acyl, sugar, phosphoryl and alkyl; oxidation and reduction of C-H, C-C, and C=C; addition and elimination of small molecules; and additionally isomerisations and ligations, etc. This allows a greater level of molecular diversity to be achieved in a dynamic combinatorial library according to the present invention than is possible with the techniques described in the prior art. Further, since many enzymes are able to function effectively under common conditions, it is also possible and sometimes desirable to carry out reaction processes with more than one enzymatic reaction at the same time. By this means it is possible to increase the diverse nature of the equilibrium mixture of compounds produced and thereby further improve the prospects of identifying entities with high binding affinity for the target of interest.

It should be understood that step (iv) does not necessarily require that the entity or entities interacting with the molecular target be identified in terms of chemical, structural or physical properties, although in many cases this may be desirable. Rather, step (iv) entails the identification of the entity or entities from the combinatorial library which interact with the molecular target. Thus, as will be apparent to a person skilled in the art, in certain cases the entity or entities interacting with the molecular target, as identified using the method of the present invention, may be isolated using conventional techniques and used in further downstream applications or processes without, or prior to, characterisation of said entity or entities. In other cases, however, it may be desirable or advantageous to analyse the entity or entities identified in step (iv) of the method according to the present invention, in order to characterise said entity or entities in terms of chemical, structural, physical or other parameters.

The method may therefore optionally further comprise the following step:

(v) characterising the entity or entities so identified in step (iv).

The entity or entities which interact with the molecular target may be characterised using any of the chemical and physical analytical methods known in the art, such as chromatographic methods, for instance HPLC, mass spectrometry, NMR, etc. The array of techniques that may be used will be apparent to a person skilled in the art.

The entity or entities produced and identified by the method of the present invention may have the same functionality or functionalities as present in the

compound or plurality of compounds, or may have modified functionalities. Thus, the method of the present invention may be used to create a dynamic combinatorial library composed of compounds that contain at least one functionality capable of binding to the molecular target, said method allowing an interchange and development of the functionalities in order to create combinations of different functionalities which interact with the molecular target in the same or different ways.

The final mixture may be analysed and the result compared to the result observed using a mixture obtained under identical conditions, but without the addition of the molecular target.

It is possible to generate dynamic combinatorial libraries according to the present invention by combining suitable substrates under conditions by which covalent bonds can easily and reversibly be formed and broken through the mediation of enzyme catalysed processes, hence enabling the creation of new and additional molecular entities and further allowing for an interchange and modification of their functionalities. The method is thus useful for creating molecules having affinity for the molecular target or other useful properties.

By allowing the compounds to reversibly react and interact and to compete for the molecular target, it is thus possible to determine which compound or moiety has an affinity for the target and eventually which compound or moiety has the best affinity for the target. This is a preferred embodiment of the present invention.

When in the context of the present invention there is referred to a "functionality", this includes simple chemical functional groups, for example, amino groups and derivatives thereof, amido groups, hydroxyl groups, carbonyl groups, carboxylate and ester groups, lactone groups, sulfonamide groups, phosphate groups, thiol groups, thiocarboxylate and thioester groups. On the other hand, this term also encompasses higher entities which themselves may contain functional groups, and non-limiting examples thereof include known pharmacophores and additionally: substituted aromatic rings, heterocycles carrying one or more heteroatoms in the ring selected from the group consisting of N, O and S, amino acids and oligo- and polypeptides, mono- and oligosaccharides and their derivatives, nucleic acid constituents and related groups.

With regard to the enzyme-catalysed modification of the compound or compounds, this may include the combination or separation of compounds.

The entity or entities produced and/or identified using the method according to the present invention may take any form. Typically, said entity or entities will have a molecular weight not greater than around 5000, preferably not greater than around 1000, and advantageously not greater than around 500. The entity or entities will possess an affinity for the molecular target, i.e. will be able to interact with the molecular target. Interaction with the molecular target may occur via the formation of a plurality of bonds, such as electrostatic and hydrogen bonds, hydrophobic interactions, etc.

With regard to the molecular target, this is preferably a molecular entity with a molecular weight typically greater than 200, most preferably greater than 500, and may be of a proteinaceous nature, such as a lipoprotein, glycoprotein and an analog of a protein, wherein the peptide bond CO-NH- may be replaced by an analogous bond, such as an imine, ester, sulfonamide, sulfone, sulfoxide, phosphate, phosphonate, phosphonamide, guanidine, urea, thiourea, or imide bond, and wherein the amino acids may be found in natural proteins or non-natural proteins. Alternatively, the molecular entity may be of a non-proteinaceous nature, such as a nucleic acid or other organic molecule having an affinity for a ligand. Such molecular entities may be in solution or adsorbed on to a solid support or optionally linked chemically to a solid support through a linker.

With regard to the enzyme, this will typically, but not exclusively, comprise a protein-based entity capable of catalysing chemical reactions and transformations, together with a cofactor where necessary, and may be natural or synthetic (mutated). In the context of this invention, the enzyme may comprise a catalytic antibody, and may further be either soluble or insoluble in the reaction medium, and may be immobilised on a support. Additionally, the enzyme may comprise other molecular entities able to carry out one or more enzymatic functions, such as molecularly imprinted polymers that are designed to enable reversible chemical transformations.

The term "reversible bond" as used herein refers to a bond in which the half time of the bond formation or cleavage under the conditions used for combinatorial library formation is typically less than one month, preferably less than one day and more preferably less than one hour.

Importantly, the compounds selected for forming the dynamic combinatorial library must comprise atoms or functional groups which allow reversible formation and

cleavage of bonds under the mediation of enzymes and hence a transformation or redistribution process resulting in an interchange of functionalities. Prerequisites that an appropriate atom or functional group has to fulfil include allowing the ready reversibility of bond formation and cleavage or transformation under mild, enzymatic conditions. These conditions should usually be compatible with the presence of the molecular target and should not ordinarily lead to modification of the target.

The present invention is dependent upon the use of enzymes to mediate the reversibility of bond formation and cleavage necessary to establish a dynamic combinatorial library of chemical compounds. Other methods of achieving reversible bond formation can optionally be used in addition to and concurrently with enzymatic methods, providing that they are compatible with the reaction conditions used and the presence of the molecular target. These can include formation of reversible bond types such as imines and their analogues, acetals, esters and lactones under hydrolytic conditions, disulphides under reductive and pH-controlled conditions, and also alkenes wherein reversibility may be rendered possible by suitable metathesis reaction conditions. This extends the concept of the present invention to include the formation of dynamic combinatorial libraries brought about by the combination of different methodologies to establish a reversible mixed equilibrium of compounds.

In carrying out the method of the present invention it may be desirable or necessary to prevent interaction of the enzyme or enzymes used to establish the dynamic equilibrium with the molecular target (for example, to prevent unwanted enzymatic modification of the molecular target). This may be achieved by physically separating the molecular target and enzymes using a selectively permeable barrier or membrane, preferably a dialysis membrane. The barrier or membrane is selected to be permeable to the relatively small molecules in dynamic equilibrium, which can therefore pass freely through the barrier or membrane, but impermeable to the relatively large enzyme(s) and molecular target.

In one preferred embodiment of the invention, the method is carried out in a reaction cell that is divided into first and second compartments separated by a suitable dialysis membrane. In the first compartment of the reaction cell there is placed the molecular target in reaction medium, whilst in the second compartment there is placed the dynamic equilibrium mixture comprising the starting compound(s) and the enzyme(s) in reaction medium. The relatively low molecular weight library

components of the dynamic equilibrium are free to diffuse across the dialysis membrane into the first compartment, thus allowing the selection and amplification of the entity or entities interacting with the molecular target, in particular the entity or entities interacting most strongly with said molecular target. The dialysis membrane is impermeable to both the enzyme(s) and the molecular target, which therefore remain separated in their respective reaction cell compartments.

When producing and utilising a dynamic combinatorial library according to the present invention, different procedures may be employed. In one procedure, library generation is performed in the presence of the target in one single step; in a slight modification to this procedure, the dynamic library is first generated and then allowed to re-equilibrate in the presence of the molecular target which is added in a subsequent step. In a second aspect, the target may be added after reversible interconversion has been halted, by, for example, removal of the enzyme(s) from the reaction system. The particulars for the above-mentioned different procedures are as follows:

A) Directed combinatorial libraries (one-step procedure): the generation of the library constituents is conducted in a single step in the presence of the molecular target, such that the library composition may adjust, leading to selection and amplification of the preferred entity or entities. Screening and selection by the molecular target occurs in parallel with the reversible generation of the library constituents. In this approach, the dynamic features of the combinatorial library are operative over the whole process.

B) Pre-equilibrated, non-directed dynamic combinatorial libraries (two-step procedure): the constituents of the library are generated by reversible interconversion and equilibration in the absence of the molecular target, which is added in a second step after reversibility has been stopped.

Thus, a second aspect of the present invention provides a method of producing a dynamic combinatorial library of compounds, comprising the following steps:

(i) providing a compound or a plurality of compounds each having at least one functionality, wherein said compound or plurality of compounds is susceptible to modification via at least one enzyme-catalysed reaction whereby such modification results in the formation of at least one entity having an arrangement of functionalities that allows it to interact with a molecular target capable of such interaction;

(ii) mixing said compound or plurality of compounds with at least one enzyme and maintaining the resulting mixture under conditions that allow reversible bond formation and cleavage;

(iii) halting reversible bond formation and cleavage;

(iv) introducing said molecular target to said mixture;

(v) identifying which entity or entities interact with said molecular target; and

(vi) optionally characterising the entity or entities so identified in step (v).

This procedure has the advantage that reversible reactions and conditions may be used which are not compatible with the presence of the molecular target to generate a diverse mixture of compounds. This process does not direct the equilibrium mixture of the library towards those entities that bind most strongly to the target and no amplification of the preferred entity or entities results. It is, however, suitable for lead generation, i.e. the discovery of compounds possessing useful properties.

Generally, the molecular target according to the present invention will comprise a protein, preferably a natural protein such as an enzyme, a receptor or an antibody or a partial structure of the foregoing. Receptors may include membrane receptors, hormone receptors, signal transducers, etc.

When the molecular target is an enzyme, the entity or entities sought to be obtained (i.e. those binding most strongly to the molecular target) may act as a substrate, an inhibitor or an activator for said enzyme.

When the molecular target is a receptor, the entity or entities sought to be obtained may act as a natural or artificial ligand, an agonist or an antagonist for said receptor.

When the molecular target is an antibody, the entity or entities sought to be obtained will comprise an antigen for said antibody.

The basic compound or compounds are selected according to their chemical structure. The molecular target plays a key role in the exploitation of dynamic combinatorial libraries through driving the formation and selection of compounds that bind thereto. In selecting suitable molecular targets for use with dynamic combinatorial library synthesis, a number of factors are considered. If the molecular target represents a protein involved in a biological process, it should be expressed, purified and post-modified in ways that allow its natural structure and activity to be maintained. The molecular target should be stable to the conditions used for library synthesis. It should

retain its desired activity, it should be able to be used in such a way that enables its removal from the reaction system, preferably with entities from the reaction mixture that bind most strongly, and its intrinsic molecular properties should be such as to allow the development of meaningful further biological assays.

Suitable enzymes for use in this invention are those used as catalysts in organic synthesis and are well known. Many are described, along with suitable substrates, in a number of texts such as 'Enzymes in Synthetic Organic Chemistry' by Wong and Whitesides, 1994, Elsevier Science Ltd. (ISBN 0 08 035942 6).

Suitable substrates may be either natural or unnatural substrates for the chosen enzymes.

A third aspect of the present invention provides the use of a method of the invention as hereinbefore described to produce a dynamic combinatorial library of compounds.

In a preferred embodiment of the present invention, the method is used to design libraries of molecules formed by reversible enzyme-catalysed hydrolytic transformations of amide and ester bonds. Such transformations are readily brought about through the use of proteases, lipases and esterases. A large number of readily available enzymes possessing relaxed substrate specificities are available and the reversible reaction conditions necessary to produce a dynamic equilibrium of compounds are readily established through the use of appropriate reaction conditions, incuding the use of solvent systems of low water activity. Suitable proteases include serine proteases, thiol proteases, metalloproteases and aspartyl proteases. Particularly suitable proteases include chymotrypsin and subtilins, elastase, pepsin, thermolysin, papain, trypsin, clostripain, endoproteases Lys-C and Glu-C, carboxypeptidases Y, B, A and M and cathepsin C. Certain of these proteases, for example serine and thiol proteases, are also useful for ester hydrolysis and transacylation reactions. The use of these classes of enzymes with appropriately functional substrates can give rise to dynamic combinatorial libraries possessing enhanced chemical diversity.

Many lipases suitable for use in the present invention are commercially available from suppliers such as Amano, Sigma, Fluka, Boehringer Mannheim and NOVO. Several possess high region- and chemo-selectivities, whereas others, such as porcine pancreatic lipase (PPL), are not usually pure and may contain other enzymes such as chymotrypsin and cholesterol esterase. Enzyme selectivity, for example for

primary alcohol ester substrates over secondary alcohol substrates, or enantioselectivity, will in such cases be determined by purity. Lipases isolated from Pseudomonas species (PSL) can be particularly selective, especially for the hydrolysis of esters of secondary alcohols and for the corresponding reverse reactions. In general, it is desirable to use lipases that have specificities related to the structure and diversity of substrates that form the initial reaction medium. In contrast to the large number of readily available lipases, relatively few 'true' esterases are commercially available. However, of those that are, pig liver esterase (PLE) and acetylcholine esterase are particularly useful in the current invention, whilst lyophilised Baker's yeast is also a valuable source of esterase activity.

In a further preferred embodiment, the method according to the present invention is used to design libraries of molecules formed by reversible C-C bond formation brought about from the action of enzymes such as aldolases on suitable donor and acceptor substrates. Many aldolases are known that catalyse the stereospecific condensation of an aldehyde with a ketone donor and all are suitable for use in the present invention. Aldolases that may be used include both Type I and Type II aldolases, such as FDP aldolase, Fuc 1-P aldolase, Rha 1-P aldolase, TDP aldolase, NeuAc aldolase, NeuAc synthetase, KDO aldolase, KDO 8-P synthetase, KHG aldolase, KDPG aldolase, KDG aldolase, DERA aldolase, TK transketolase, TA transaldolase, oxynitrilases, pyruvate decarboxylase, acetyl transferases such as acetyl CoA, cyclaese, such as lanosterol cyclase. Suitable acceptor substrates may be natural substrates or unnatural substrates and include aldehydes and ketones, which further include aliphatic and aromatic aldehydes, substituted aldehydes, and monosaccharides and oligosaccharides and their derivatives. Substrates may optionally be phosphorylated. Suitable donor substrates may also be natural or unnatural and include: dihydroxyacetone phosphate, glyceraldehydes-3-phosphate, fructose-1,6-diphosphate, pyruvate, phosphoenolpyruvate, pyruvate analogues, aldehydes such as acetaldehyde and proprionaldehyde, and amino acids such as serine.

In a still further preferred embodiment, the method according to the present invention is used to design libraries of molecules formed by reversible glycosidic bond formation. It is well known that oligosaccharides and polysaccharides are found in nature as components of a broad range of molecular structures. Oligosaccharides and polysaccharides play vital roles in cellular communication processes and as points of

attachment for bacteria, viral particles, antibodies and other proteins. In addition, carbohydrates are being increasingly developed as an important class of pharmaceutical agents. Nature uses a number of different groups of enzymes in the biosynthesis of oligosaccharides, such as glycosyl transferases and transglycosidases. Glycosyl transferases require sugar monomers to be phosphorylated prior to condensation with an acceptor species and may be highly selective with respect to substrate and the type of glycosidic bond formed. Glycosidases, a class of enzymes that function *in vivo* to cleave glycosidic bonds through hydrolysis, can be used under appropriate conditions as synthetic catalysts. In general, glycosidases are less specific when compared to glycosyl transferases. Each of these classes of enzymes may be used under defined conditions to establish reversible glycosidic bond formation and thus have utility in the production of dynamic combinatorial libraries. The use of glycosyl transferases usually requires the additional presence of phosphorylating enzymes to produce the desired activated donor substrates *in situ*. More particularly preferred, owing to their ease of use, are the glycosidases. It is well known that such enzymes may be used under thermodynamic and kinetic control and that under thermodynamic conditions equilibrium between synthesis and catabolism may be manipulated through the use of water immiscible co-solvents and varying substrate and acceptor concentrations. In addition, regioselective reaction of one hydroxyl group of an unprotected sugar with a glycosyl donor can be achieved, and further, regioselectivity can be varied through the use of glycosidases of different origin. Where such enzymes are being used to provide dynamic combinatorial libraries, it will be appreciated that regioselectity is not always essential or even desirable, and that selection of an appropriate equilibrium product through binding with a target will drive the equilibrium mixture towards this preferred product. Reversible glycosyl transfer to non-sugar acceptors is also readily achieved through the use of glycosidases. Typical acceptors are alcohols, which may be mono- or poly-functional with respect to the numbers of hydroxyl groups possessed, and amines, to form O-linked and N-linked glycosides respectively. Glycosidases may also be combined with other enzymes, including glycosyl transferases, kinases and transglycosidases in order to provide the rapid synthesis of more highly functional dynamic combinatorial libraries.

In a particularly preferred embodiment of the present invention, the method is used to establish a dynamic combinatorial library for the binding of carbohydrates to

those proteins belonging to the class of lectins. One example of a lectin is Concanavalin A (Con A) which is specific for a branched trimannoside core unit, located in N-glycosidic carbohydrate-peptide linkages of glycoproteins often associated to cell surfaces. For this reason, Con A is extensively used as a tool in histochemical staining.

The invention will now be described further in the following non-limiting examples.

Example 1

General procedure

N-acetyl neuramic acid aldolase (EC 4.1.3.3, cat. 153493, 22.2Umg-1) and wheat germ agglutinin (cat. 152266, lyophilised powder) were purchased from ICN Biomedicals.

The following stock solutions were prepared in 0.05M KPi buffer, pH 7.5:

Aldolase stock, 20UmL-1;

Wheat germ agglutinin stock, 50mgmL-1;

Sugar stock solutions containing 0.5mgmL-1 sodium azide, 0.1mmolmL-1, sodium pyruvate, and 0.05mmolmL-1 each of manNAc (solution A), manNAc and mannose (solution B), manNAc, mannose and lyxose (solution C), or mannose and lyxose (solution D).

Incubation mixtures A-D contained 10μL enzyme stock, 20μL WGA stock, and 10μL sugar stock A, B, C or D respectively. Controls contained 10μL enzyme stock, 20μL 0.05M KPi buffer, and 10μL sugar stock A, B, C, or D. After centrifugation (1min at 3000rpm) the solutions were incubated at 37oC without stirring. At intervals 5μL aliquots were withdrawn, diluted to 500μL with milliQ water, and immediately analyzed by HPLC.

Results

Figures are area counts from HPLC traces. The two- and three-component systems both clearly show significant shifts in product distribution relative to controls.

It seems that the control systems reach equilibrium relatively quickly (overnight), and the lectin-containing systems initially approach a similar equilibrium, then slowly begin to deviate. That seems consistent with the low concentrations (<5mmolar) of sialic acid produced; binding to the lectin only becomes significant near

maximum sialic acid concentration (close to equilibrium), and subsequent shifts are consequently slow.

In the 3-component system KDN and KDO are not suppressed to the same extent. This might indicate binding to the lectin to some extent, protecting the better binder somewhat from the reverse reaction. It might also indicate that the KDO -> lyxose reaction is faster than the KDN -> man reaction; if there is no thermodynamic distinction between the aldolase products then the perturbation caused by the lectin can be redressed at the expense of either product, and the faster reacting species will go first. Mixture D showed no change in product ratio over the first two days, which covers most of the bases as far as control experiments go. We have also shown re-equilibration of a mixture of sialic acid and mannose to sialic acid, KDN, sugars, and pyruvate, which certainly confirms that the reactions are reversible.

Table 1

Mixture A – manNAc -> sialic acid

	control (16hrs)	16hrs	40hrs	112hrs
manNAc	24151111	45964119	46331690	19642472
pyruvate	7880588	7720074	10321647	7028816
sialic acid	9075209	9832980	13113214	9614538
sialic/manNAc	0.38	0.21	0.28	0.49
sialic/pyruvate	1.15	1.27	1.27	1.37

Table 2

Mixture B – manNAc + man -> sialic acid + KDN

	control (16hrs)	16hrs	40hrs	112hrs
sugars	42920475	74204745	47662994	20112993
pyruvate	3746990	4994598	4415700	4325470
sialic acid	4577318	7016243	5629618	6151075
KDN	4664090	6550985	3830385	363315
sialic/sugars	0.11	0.09	0.12	0.31
sialic/pyruvate	1.22	1.40	1.27	1.42
sialic/KDN	0.98	1.07	1.47	16.9

See Fig. 1

Table 3

Mixture C – manNAc + man + lyxose -> sialic acid + KDN + KDO

	control (16hrs)	16hrs	40hrs	112hrs
sugars	84929780	99742212	82100774	55073310
pyruvate	6652231	6838271	8584363	10838392
sialic acid	7505701	8469833	9872545	12998891
KDN	12183339	13135403	14170583	15837586
KDO	7944589	8088977	7639395	3175524
sialic/sugars	0.09	0.08	0.12	0.24
sialic/pyruvate	1.13	1.24	1.15	1.20
sialic/KDN	0.62	0.64	070	0.82
sialic/KDO	0.94	1.05	1.29	4.09
KDN/KDO	1.53	1.62	1.86	4.99

Table 4

Mixture D – man + lyxose -> KDN + KDO

	control (16hrs)	16hrs	40hrs	112hrs
sugars	51732035	84813089	80794064	
pyruvate	4097770	4786740	5330683	
KDN	9469652	11198119	11846923	
KDO	5362450	6306756	6654458	
KDN/sugars	0.18	0.13	0.15	
KDN/pyruvate	2.31	2.34	2.22	
KDN/KDO	1.77	1.78	1.78	

CLAIMS

1. A method of producing a dynamic combinatorial library of compounds in the presence of a molecular target capable of interaction with at least one functionality, said method comprising the following steps:

   (i) providing a compound or a plurality of compounds each having at least one functionality, wherein said compound or plurality of compounds is susceptible to modification via at least one enzyme-catalysed reaction whereby such modification results in the formation of at least one entity having an arrangement of functionalities that allows it to interact with said molecular target;

   (ii) mixing said compound or plurality of compounds with at least one enzyme and maintaining the resulting mixture under conditions that allow reversible bond formation and cleavage;

   (iii) introducing said molecular target to said mixture and further maintaining said mixture under conditions that allow an equilibrium redistribution in favour of the entity or entities interacting with said molecular target; and

   (iv) identifying which entity or entities interact with said molecular target.

2. A method according to claim 1, wherein the method further comprises the following step:

   (v) characterising the entity or entities so identified in step (iv).

3. A method according to either of claims 1 or 2, wherein step (iii) is carried out simultaneously with step (ii).

4. A method according to either of claims 1 or 2, wherein step (iii) is carried out after step (ii).

5. A method according to any one of claims 1 to 4, wherein the at least one functionality comprises a simple chemical functional group.

6. A method according to claim 5, wherein the simple chemical functional group is selected from amino groups and derivatives thereof, amido groups, hydroxyl groups, carbonyl groups, carboxylate groups, ester groups, lactone groups, sulfonamide groups, phosphate groups, thiol groups, thiocarboxylate groups and thioester groups.

7. A method according to any one of claims 1 to 4, wherein the at least one functionality comprises a complex entity.

8. A method according to claim 7, wherein the complex entity is selected from pharmacophores, substituted aromatic rings, heterocycles carrying one or more heteroatoms in the ring selected from the group consisting of N, O and S, amino acids, oligo- and polypeptides, mono- and oligosaccharides and derivatives thereof, and nucleic acid constituents.

9. A method according to any one of claims 1 to 8, wherein the entity or entities interacting with said molecular target have a molecular weight not greater than around 5000.

10. A method according to claim 9, wherein the entity or entities interacting with said molecular target have a molecular weight not greater than around 1000.

11. A method according to claim 10, wherein the entity or entities interacting with said molecular target have a molecular weight not greater than around 500.

12. A method according to any one of claims 1 to 11, wherein the molecular target has a molecular weight greater than 200.

13. A method according to claim 12, wherein the molecular target has a molecular weight greater than 500.

14. A method according to any one of claims 1 to 13, wherein the molecular target is of a proteinaceous nature.

15. A method according to claim 14, wherein the molecular target is selected from a lipoprotein, glycoprotein and a protein analogue in which a peptide bond CO-NH- is replaced by a bond selected from an imine, ester, sulfonamide, sulfone, sulfoxide, phosphate, phosphonate, phosphonamide, guanidine, urea, thiourea or imide bond.

16. A method according to claim 14, wherein the molecular target is selected from an enzyme, receptor or antibody or partial structures thereof.

17. A method according to any of claims 1 to 13, wherein the molecular target is of a non-proteinaceous nature.

18. A method according to claim 17, wherein the molecular target comprises a nucleic acid.

19. A method according to any one of claims 1 to 18, wherein the molecular target is in solution.

20. A method according to any one of claims 1 to 18, wherein the molecular target is immobilised on a support.

21. A method according to any one of claims 1 to 20, wherein the enzyme is selected from natural or mutated protein-based entities.

22. A method according to claim 21, wherein the enzyme is a catalytic antibody.

23. A method according to claim 21, wherein an enzyme cofactor is provided.

24. A method according to any one of claims 1 to 20, wherein the enzyme comprises a molecularly imprinted polymer designed to enable a reversible chemical transformation.

25. A method according to any one of claims 1 to 24, wherein the enzyme is in solution.

26. A method according to any one of claims 1 to 24, wherein the enzyme is immobilised on a support.

27. A method according to any one of claims 1 to 26, wherein the method incorporates a further means of achieving reversible bond formation

28. A method according to any one of claims 1 to 27, wherein interaction of the at least one enzyme and the molecular target is prevented.

29. A method according to claim 28, wherein interaction of the at least one enzyme and the molecular target is prevented using a selectively permeable barrier.

30. A method according to claim 29, wherein the selectively permeable barrier comprises a dialysis membrane.

31. A method of producing a dynamic combinatorial library of compounds, comprising the following steps:

(i) providing a compound or a plurality of compounds each having at least one functionality, wherein said compound or plurality of compounds is susceptible to modification via at least one enzyme-catalysed reaction whereby such modification results in the formation of at least one entity having an arrangement of functionalities that allows it to interact with a molecular target capable of such interaction;

(ii) mixing said compound or plurality of compounds with at least one enzyme and maintaining the resulting mixture under conditions that allow reversible bond formation and cleavage;

(iii) halting reversible bond formation and cleavage;

(iv) introducing said molecular target to said mixture;

(v) identifying which entity or entities interact with said molecular target; and

(vi) optionally characterising the entity or entities so identified in step (v).

32. A method according to claim 31, wherein reversible bond formation and cleavage is halted by removal of the at least one enzyme.

33. A method according to claim 1, wherein the combinatorial library comprises a library of molecules formed by reversible enzyme-catalysed hydrolytic transformations of amide and ester bonds.

34. A method according to claim 33, wherein the at least one enzyme comprises a protease selected from serine proteases, thiol proteases, metalloproteases and aspartyl proteases.

35. A method according to claim 33, wherein the at least one enzyme comprises a protease selected from chymotrypsin, subtilins, elastase, pepsin, thermolysin, papain, trypsin, clostripain, endoproteases, Lys-C, Glu-C, carboxypeptidases Y, B, A and M, and cathepsin C.

36. A method according to claim 33, wherein the at least one enzyme comprises a lipase selected from porcine pancreatic lipase and lipases isolated from Pseudomonas species.

37. A method according to claim 33, wherein the at least one enzyme comprises an esterase selected from pig liver esterase, acetylcholine esterase and lyophilised Baker's yeast.

38. A method according to claim 1, wherein the combinatorial library comprises a library formed by reversible enzyme-catalysed C-C bond modification.

39. A method according to claim 38, wherein the at least one enzyme comprises an aldolase selected from FDP aldolase, Fuc 1-P aldolase, Rha 1-P aldolase, TDP aldolase, NeuAc aldolase, NeuAc synthetase, KDO aldolase, KDO 8-P synthetase, KHG aldolase, KDPG aldolase, KDG aldolase, DERA aldolase, TK transketolase, TA transaldolase, oxynitrilases, pyruvate decarboxylase, acetyl transferases, and cyclases.

40. A method according to either of claims 38 and 39, wherein the compound or plurality of compounds includes a compound selected from aliphatic and

aromatic aldehydes, substituted aldehydes, ketones, monosaccharides and oligosaccharides.

41. A method according to any one of claims 38 to 40, wherein the compound or plurality of compounds includes a compound selected from dihydroxyacetone phosphate, glyceraldehydes-3-phosphate, fructose-1,6-diphosphate, pyruvate, phosphoenolpyruvate, pyruvate analogues, aldehydes, and amino acids.

42. A method according to claim 1, wherein the combinatorial library comprises a library formed by reversible enzyme-catalysed glycosidic bond formation.

43. A method according to claim 42, wherein the at least one enzyme is selected from glycosyl transferases and glycosidases.

44. A method according to claim 1, wherein the combinatorial library comprises a library of carbohydrates that bind to lectins.

45. The use of a method according to any one of claims 1 to 44 to produce a combinatorial library of compounds.

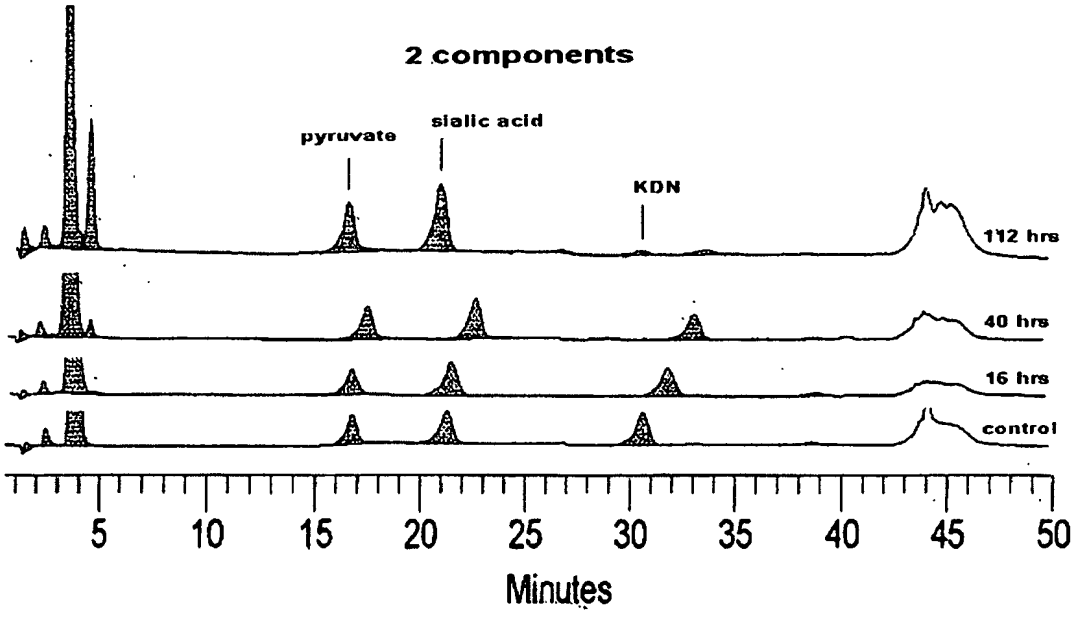
2 components
pyruvate
sialic acid
KDN
112 hrs
40 hrs
16 hrs
control
5
10
15
20
25
30
35
40
45
50
Minutes

FIG. 1

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 03/01615

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01J19/00 C07B61/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B01J C07B G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BURGER M T ET AL: "Enzymatic, polymer-supported formation of an analog of trypsin inhibitor A90720A: a screening strategy for macrocyclic peptidase inhibitors" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 119, no. 51, 24 December 1997 (1997-12-24), pages 12697-12698, XP002164640 ISSN: 0002-7863 page 12697, left-hand column, line 8 -page 12698, right-hand column, line 20; figure 1 --- -/--	1-27, 31-45

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2003	22/08/2003

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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